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RESEARCH ARTICLE

What is the optimal anesthetic protocol for measurements of cerebral autoregulation in spontaneously breathing mice?

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Abstract Autoregulation, an important feature of the cerebral circulation, is affected in many diseases. Since genetically modified mice are a fundamental tool in biomedical research, including neuro(bio)logy also in this specie measurements of cerebral autoregulation (CA) are mandatory. However, this requires anesthesia that unfortunately significantly impacts cerebral perfusion and consequently might distort CA measurements directly or by altering arterial pCO_2. The latter can be avoided by artificial ventilation but requires several control measurements of blood gases, each consuming at least 100 µl of blood or 5% of a mouse's blood volume. To avoid such diagnostic hemorrhage, we systematically analyzed the effect of different common anesthetic protocols used for rodents in spontaneously breathing mice on CA measured with Laser speckle perfusion imaging. Halothane, Isoflurane and Pentobarbital abrogated CA and Ketamin/Xylazine as well as Chloralose had a moderate reproducibility. In contrast, the rather rarely used anesthetic Ethomidate applied in low doses combined with local anesthetics had the best reproducibility. Although with this anesthesia the lower CA limit was lower than with Ketamin/Xylazine and Chloralose as reported in the handful of papers so far dealing with CA in mice, we suggest Ethomidate as the anesthetic of choice for CA measurements in spontaneously breathing mice.

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Introduction

Normally, in the brain, perfusion and local metabolic demand are closely linked (Kuschinsky 1990, 1991), and as long as the metabolic demand of the brain remains constant, a homeostatic regulatory mechanism called cerebral auto-regulation (CA) allows the cerebral blood flow (CBF) to remain relatively constant during variations in arterial blood pressure (Paulson et al. 1990). When CA is abolished, fluctuations of arterial blood pressure are accompanied by either passive reduction or increase in CBF, leading to syncope and falls, severe cerebral ischemia and edema (Faraci and Heistad 1998). Mostly, disturbed CA is due to chronic hypertension (Immink et al. 2004) resulting in higher CA limits thereby making chronic hypertensive patients highly vulnerable to brain ischemia in response to unforeseen hypotension (Strandgaard 1976). Although the upward shift of the CA limits in chronic hypertension has been described many years ago (Vorstrup et al. 1984; Harper 1987; Werber et al. 1990), its cause(s) are still poorly understood. However, genetically modified mice raise new options to answer also this question. Thus, there is a certain need for a reliable technique to measure CA in mice.

Measurements of the CBF response to blood pressure changes consider either dynamic or static CA (Panerai 2008). Dynamic CA is a transient response to spontaneous fluctuations in systemic arterial blood pressure, changes in posture or other maneuvers resulting in a sudden blood pressure change. In man, dynamic CA can be assessed with

transcranial Doppler ultrasound and (noninvasive) blood pressure recordings together with wavelet analysis (Novak et al. 2004; Hu et al. 2008; Lo et al. 2008). Unfortunately, such a technique is not available for mice. In rodents including mice, dynamic CA can be assessed by carotid clamping (Rosengarten et al. 2006; Schubert et al. 2008). Static CA is defined as the steady-state relationship between CBF and mean arterial blood pressure characterized in humans by a constant CBF between mean arterial blood pressures of approximately 50 and 150 mmHg. Static CA is measured in experimental animals using Laser Doppler flowmetry after exposure of the skull or brain surface together with manipulations of the blood pressure by controlled bleeding (until complete exsanguination) sometimes combined with phenylepinephrine infusion via catheters inserted to the femoral vessels. It is obvious that such measurements require sufficient anesthesia. However, anesthetics themselves influence the reactivity of the brain arterioles either directly e.g. volatile anesthetics dilate arteries due to attenuation of Ca^{2+} entry through voltage-gated Ca^{2+} channels on vascular smooth muscle cells (Bosnjak et al. 1992) or, especially in spontaneously breathing animals, indirectly by respiratory depression that increases arterial pCO_2. Brain vessels are very sensitive to CO_2, and therefore, increased pCO_2 values are accompanied by cerebral vasodilatation (Kuschinsky 1997) and failure of CA. The impact of the arterial pCO_2 becomes even more obvious when considering reports showing that the CA disturbed by Isoflurane anesthesia could be improved by hypocapnia (McCulloch et al. 2005). Although the CO_2 problem can be circumvented by artificial ventilation of the animal, the latter introduces an additional trauma and lengthens surgery. More importantly, the adjustment of the respirator requires several blood gas analyses, each of it consuming at least 100 µl blood. In larger animals, such as rats, this is no problem, but in mice 100 µl equals about 5% of the total blood volume. In mice blood loss, prolonged operations and increased operative trauma per se easily distort the blood acid base status, most evident from a drop in base excess (own unpublished observations). The reduced base excess is either accompanied by a drop in arterial pH and/or compensatory decrease of the arterial pCO_2. Since pial arteries are also sensitive to hydrogen ions (Kuschinsky 1982), both changes again influence either way the constriction state of brain arterioles.

The present study aimed to systematically evaluate different commonly used anesthetic protocols on static CA in spontaneously breathing mice. Specifically, for measurement of CA in rats Barbiturates (Merzeau et al. 2000; Paterno et al. 2000), volatile anesthetics such as Halothane (Verhaegen et al. 1993; Pedersen et al. 2003) or Isoflurane (Ayata et al. 2004; Tonnesen et al. 2005), Ketamine/Xylazine, Chloralose urethane (Niwa et al. 2002) or Chloralose alone (Ayata et al. 2004; Rosengarten et al. 2006) have been used. In general, in the mentioned studies, the rats were artificially ventilated. In a previous study with a setting that is very sensitive to impaired cerebral vasoreactivity on spontaneously breathing rats, we were using Ethomidate (Vogel and Kuschinsky 1996) that is known to have relatively little effect on CBF (Janssen et al. 1975; Famewo and Odugbesan 1978). The present study shows that Ethomidate provides the most consistent measurements, appears to preserve the cerebral vasoreactivity best and is thus recommended as the anesthetic of choice for CA measurements in spontaneously breathing mice.

Methods

All experiments were performed on C57Bl6 mice and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and institutional guidelines and were approved by the Cantonal Veterinary Department, Zurich, Switzerland. Since there was no gender difference for any anesthetic protocol, mice of both genders were used for the study except for telemetric blood pressure and heart rate measurements where only males were used.

Generally, the same anesthetic was used for surgery and the subsequent experiments. Since in case of Ethomidate this is not possible, surgery was performed during Isoflurane anesthesia (2–2.5%) and the animals were allowed to adapt for at least 30 min after discontinuing Isoflurane and commencing the Ethomidate infusion before the CA measurements were started. All anesthetic protocols are summarized in Table 1. During surgery and the subsequent experiment, the body temperature of the animals was kept constant at 37°C using a temperature controlled heating pad.

After induction of anesthesia, the mice were equipped with catheters in both femoral arteries and veins for the measurement of blood pressure, heart rate (PlugSys, Hugo Sachs Electronics, Germany and PowerLab, ADInstruments, Germany), blood gases and acid–base status (AVL700, Radiometer Medical, Denmark) and hematocrit and the infusion of anesthetics (cf. Table 1) and phenylepinephrine. Before closing the wounds, a drop of 2% Lidocain (Streuli, Switzerland) was applied to the wound. Thereafter the mice were placed in a stereotactic frame, the scull was exposed and flushed with 2% Lidocain. Then a Laser speckle perfusion imager (moorFLPI, Moor Instruments, UK) was adjusted to its maximal magnification and five regions of interest (ROI's, 3 on the left and 2 on the right hemisphere) were defined avoiding visible vessels. In accordance with others (Ayata et al. 2004), preliminary

Table 1 Anesthetic protocols

Active component	Trade name	Provider	Route	Initial dosage	Maintenance dosage
Ethomidate	Ethomidate Lipuro	B. Braun Medical AG, Sempach, Germany[a]	i.v.	80 µg/min for 3 min	14–26 µg/min
Ketamine/Xylazine	Rompun/ Narketan	Ratiopharm, Ulm, Germany/Bayer, Leverkusen, Germany	i.p.	90 mg kg^{-1}/ 9 mg kg^{-1}	40 mg kg^{-1} every 30 min/4 mg kg^{-1} every 30 min
Alpha-chloralose		Siqma-Aldrich GmbH, Buchs, Switzerland[b, c]	i.p./i.v.	25 mg kg^{-1}	75 mg kg^{-1} h^{-1}
Isoflurane	IsoFlo	Abbott AG, Baar, Switzerland	Inhalation	5%	1.50%
Haiotnane	Halothane	Arovet AG, Zollikon-Station, Switzerland	Inhalation	4%	0.90%
Pentobarbital	Vetanarcol	Veterinaria AG, Zurich, Switzerland[c]	i.p./i.v	100 mg kg^{-1}	1 mg kg^{-1} h^{-1}

[a] After finishing the preparation during Isoflurane anesthesia

[b] 16.6 mg Chloralose dissolved together with 25 mg sodiumtetraborate * $10H_2O$/ml H_20

[c] After finishing the preparation changed to i.v. application

experiments have shown that the transparency of the scull of mice is high enough to not reduce significantly the laser signal as long as it is kept wet with artificial cerebrospinal fluid or 0.9% saline warmed to 37°C. The images were acquired at 25 Hz and the traces of the ROI's were sampled with a time constant of 0.5 s. During surgery and the subsequent measurements of static CA, animals were allowed to breathe pure oxygen spontaneously.

At the very beginning of the recordings, a screwdriver handle was dropped onto the arterial line connected to the blood pressure transducer. This produced an artifact simultaneously in both the blood pressure and the laser perfusion trace that was later used to synchronize both traces for offline calculation of the CA limits. Then the mice were slowly exsanguined via the second arterial line while continuously recording the laser speckle perfusion and blood pressure signal. The exsanguination rate was adjusted manually by continuous inspection of the blood pressure trace to get a linear and constant decrease of the blood pressure (about 1 mmHg/min, total exsanguination time: 35–40min). In addition, some animals anesthetized with Ethomidate, Chloralose or Ketamine/Xylazine received an infusion of phenylepinephrin (Sintetica, Switzerland, 1 ng/g/min) that was started 3 min before starting the CA measurement and lasted until its end.

For determination of the CA limits, all laser speckle perfusion and blood pressure values before the marker artifact were discarded and then the data were averaged in ten-second intervals using macros written in Excel (Microsoft). The laser speckle perfusion values obtained this way were then plotted as a function of the corresponding blood pressure values. Then the lower CA limit was determined by linear extrapolation by starting with the lowest blood pressure values and the upper limit in a similar way by starting with the highest blood pressure values as described elsewhere for the "plateau constraint" (Pedersen et al. 2003). This was done separately for each of the five ROI's. The final CA limits for each animal were defined as the mean of those determined for each ROI, and these mean values were used for statistical analysis. The intra-individual variation of the ROI's was very low (coefficient of variation: 1.3–2.8%).

Another experimental group was equipped with telemetric blood pressure sensors (TA11PA-C10 transmitter, Data-Sciences International, USA) as described recently (Schuler et al. 2009, 2010) to assess the effect of anesthesia on arterial blood pressure and heart rate in comparison with conscious animals. In an additional control experiment, the effect of switching from normal air to pure oxygen on Laser speckle imaging during Isoflurane and Ethomidate anesthesia has been assessed.

Data are presented as means ± standard deviation and were analyzed with the GraphPad PRISM 4 Software (version 4.01) using ANOVA and Students *t* test or Kruskal–Wallis test (in case the number of values were not equal for all groups) for unpaired samples with Bonferroni's or Dunn's post hoc test, respectively. *P* values of <0.05 were considered significant.

Results

Table 2 shows the physiologic variables of all naïve animals used for the study. The blood gas values of mice treated with phenylepinephrine were similar, but mean arterial blood pressure as well as heart rate were considerably higher by 53 and 61%, respectively (not shown). The most striking difference between the groups of naïve mice was found concerning the arterial pCO_2 with highest values in Pentobarbital and Ketamine/Xylazine anesthetized mice and lowest during Isoflurane anesthesia. In addition, heart rate

Table 2 Physiologic variables

	Weight (g)	pH	$pC0_2$ (mmHq)	pO_2 (mmHg)	BE (mmol/L)	HCT	Mean arterial blood pressure (mmHg)	Heart rate (min^{-1})	Anesthesia (abbr.)	*n*
Mean	31.00	7.44	36.71	369.00	1.58	43.32	81.52	407.B1	Ethomidate (E)	6
SD	3.79	0.06	7.07	46.62	2.20	3.05	5.90	84.40		
Mean	27.00	7.17*	53.98$^{\&}$	486.28	−4.37	43.17	92.03	248.14*	Ketamine/ Xylazine (K)	6
SD	4.10	0.02	8.62	131.67	3.86	1.22	7.79	42.12		
Mean	35.00*	7.43	40.43	366.36	3.30*	40.60	86.73	467.75	Chforaiose (C)	6
SD	2.83	0.05	5.51	145.64	2.87	1.38	12.55	76.22		
Mean	25.17	7.46	27.38	383.28	−4.77$^{\#}$	47.94	94.82$^{\#}$	430.00	Isofluran 1.2% (I)	6
SD	1.47	0.04	5.65	89.02	2.47	4.34	3.30	54.25		
Mean	27.50	7.29$^{\#}$	46.80/*	389.96	−3.43	44.50	84.40	402.55	Halothane 0.9% (H)	6
SD	4.85	0.03	4.12	124.03	1.07	1.49	4.69	60.70		
Mean	24.40	7.18*	69.14*	439.71	−3.92	43.10	78.93	304.93$^{\#}$	Pentobarbital (P)	5
SD	0.89	0.09	9.85	65.04	4.26	2.04	4.96	20.43		
Mean	25.68					42.87	104.19*	506.34	Telemetry (conscious) (T)	6
SD	1.01					3.82	2.33	49.06		
	$*P < 0.05$ vs. K, I, H, P, T	$*P < 0.05$ vs. I, H, E, C $^{\#}P < 0.05$ vs. I, E, C	$*P < 0.01$ vs. I, H, E, C $^{\&}P < 0.01$ vs. I, E, C $^{\#}P < 0.01$ vs. I	ns	$*P < 0.05$ vs. K, I, H, P $^{\#}P < 0.05$ vs. E	ns	$*P < 0.01$ vs. E, C, H, P $^{\#}P < 0.05$ vs. E, P	$*P < 0.01$ vs. E, C, I, H, T $^{\#}P < 0.01$ vs. C, T		

is lower in those anesthetic regimes that result in respiratory depression such as Ketamine/Xylazine and Pentobarbital. Most likely this is an effect of hypercapnia that has been shown to induce bradycardia in mice (Campen et al. 2004). The high pO_2 values in all experimental groups are due to pure oxygen breathing. The telemetric blood pressure and heart rate data had been obtained in the context of a recent study (Schuler et al. 2010). In comparison with these data, all anesthetics reduced blood pressure and heart rate. Switching from air to pure oxygen did not affect the blood pressure or the Laser speckle signal (data not shown).

Figure 1 shows example autoregulation curves (mean of all ROIs of a single animal) of all anesthetics tested without parallel phenylepinephrine infusion. Halothane as well as Isoflurane resulted in complete abrogation of CA that is for Isoflurane in agreement with previous reports (Ayata et al. 2004). A bit surprisingly, since also used in several studies dealing with CA, Pentobarbital anesthesia also was associated with a complete loss of CA. This is most probably due to the anesthesia-induced hypercapnia of the spontaneously breathing mice (cf. Table 2).

In Ketamine-/Xylazine- and Chloralose-anesthetized mice, the lower CA limit was around 44 and 53 mmHg, respectively, which is in agreement with previous reports on mice investigated using Chloralose (Ayata et al. 2004). In contrast, the lower CA limit in Ethomidate-anesthetized mice was considerably lower at around 25 mmHg and the drop was more abrupt than in Ketamine-/Xylazine- or Chloralose-anesthetized mice (cf. Fig. 1). However, in naïve animals, the differences in lower limit of CA reached statistical significance only for the comparison between Ethomidate and Chloralose. For Ethomidate, Ketamine/Xylazine and Chloralose, the lower limit was also determined during phenylepinephrine infusion resulting in the same limits (Fig. 2); however, between these groups, none of the differences were statistically significant. This is most likely due to much larger data scatter in Ketamine/Xylazine and

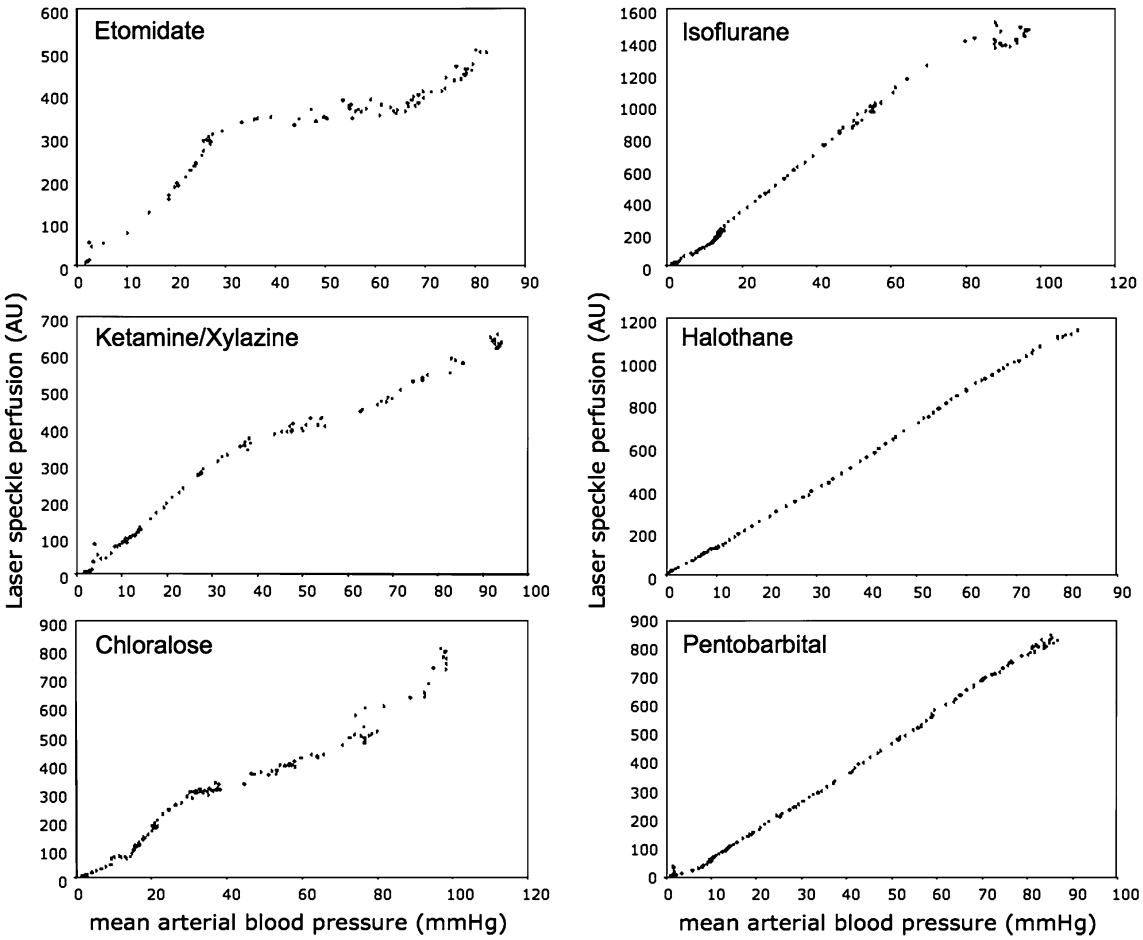

Fig. 1 Representative examples of autoregulation curves (mean of all five ROI's of a single animal) obtained with the different anesthetics as indicated. The anesthetics shown on the *right* completely abrogated cerebral autoregulation (CA). For Ethomidate, the range of CA is largest. Also in Ketamine-/Xylazine-anesthetized mice, the lower as well the upper CA limit can be seen, whereas in Chloralose-anesthetized mice, the upper limit is not visible since with falling blood pressure cerebral perfusion is reduced rather in an exponential fashion until the lower CA limit is reached

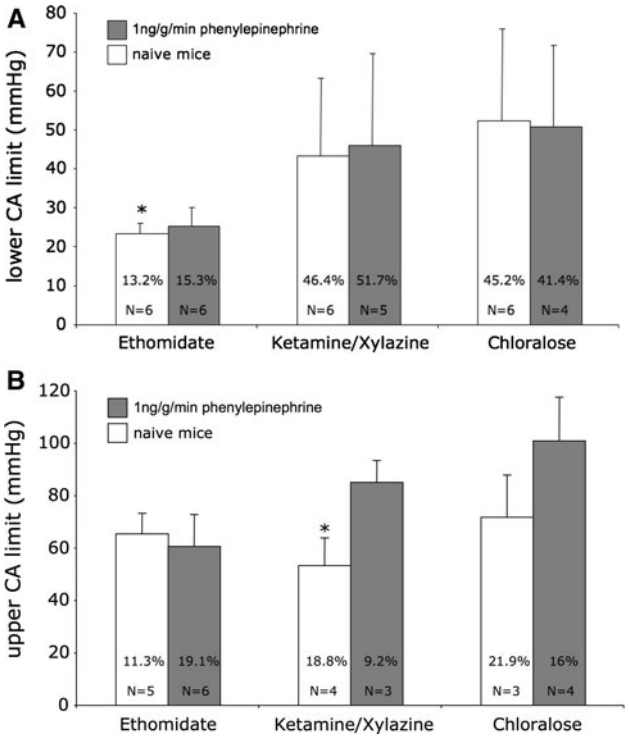

Fig. 2 Mean lower (**a**) and upper (**b**) CA limits determined mice with and without phenylepinephrine treatment. **a** The lower CA limit was the same with or without phenylepinephrine treatment but lowest during Ethomidate anesthesia. However, this was statistically significant only compared to Chloralose (both untreated, $*P < 0.05$), most likely due to the high data scatter in the Ketamine/Xylazine and Chloralose groups as indicated by the coefficient of variation (numbers in the first row). The more than 3-times smaller coefficient of variation indicates a better reproducibility during Ethomidate anesthesia compared to both, Ketamine/Xylazine and Chloralose. **b** Determination of the upper limit was hampered by the fact that some traces showed an exponential shape above the lower CA limit (cf. Fig. 1, Chloralose). In keeping with this limitation, the upper CA limits in Ketamine-/Xylazine- and Chloralose-anesthetized animals tended to be higher in case of phenylepinephrine treatment in contrast to Ethomidate-anesthetized mice. In Ketamine-/Xylazine- and Chloralose-anesthetized mice, the coefficient of variation was lower than that for the lower CA limit and in Ethomidate-anesthetized mice similar to that of the lower limit. $*P < 0.05$ vs. Chloralose + phenylepinephrine, means ± SD, N = number of animals per group

Chloralose anesthetized mice compared to those anesthetized with Ethomidate. Accordingly the coefficient of variation was between 3- and 3.9-times lower during Ethomidate anesthesia than during Ketamine/Xylazine and Chloralose anesthesia. These latter data indicate that Etomidate has the best reproducibility concerning the measurement of the lower CA limit.

Regarding the upper limit in some animals of all anesthetic protocols, the determination was not possible since above the lower limit the CA curves showed an exponential shape. This was even more the case in phenylepinephrine-treated mice. However, the best detection rate of an upper limit was found in Ethomidate-anesthetized mice. In contrast to the lower limit, there were no major differences in the upper limit between naïve mice anesthetized with Ethomidate, Ketamine/Xylazine or Chloralose. In phenylepinephrine-treated mice, the upper limit tended to be higher during Ketamine/Xylazine and Chloralose anesthesia. In contrast to the lower limit, the measurements of the upper limit appeared to be more stable (maximum coefficient of variation of about 22%) irrespective of the fact that sometimes the upper limit could not be determined at all.

By taking the measurements of the lower and upper limits together in naïve Ethomidate anesthetized mice, the CA range was considerably larger (42 mmHg vs. 9.9 and 19.2 mmHg during Ketamine/Xylazine and Chloralose anesthesia). During phenylepinephrine treatment, the differences between lower and upper limit were quite similar (34.6, 35.6 and 36.8 mmHg). The latter data however are based only on a few mice and thus should be taken with caution.

In addition, we aimed to assess the vasodilatation induced by the anesthetics themselves as well as the vasodilator capacity (defined as the difference between the initial and maximum perfusion per mmHg) in response to the falling blood pressure during the different anesthetic protocols. Although Laser speckle perfusion imaging does provide only semi-quantitative data, the measurements are quite stable as determined with a motility standard provided by the manufacturer of the imager along with the device. Thus, we calculated the initial Laser speckle perfusion/mmHg before exsanguination was started. In addition, we calculated the difference between the initial perfusion/mmHg and the maximal perfusion/mmHg before the final drop of the cerebral perfusion. In case there was no increase observed from the initial value, the perfusion/mmHg value at a blood pressure of 50 mmHg was taken. Figure 3 shows that there were large differences in the initial perfusion between the different anesthetics with a maximum factor of nearly 3 between Ethomidate and Halothane. Note that Halothane, Isoflurane, and Pentobarbital, which abrogated CA, exhibit the highest initial laser speckle perfusion as well as perfusion/mmHg values. This indicates anesthesia-induced vasodilatation. In line with this, the maximal increase in perfusion/mmHg as a result of the falling blood pressure was found also in Ethomidate-anesthetized mice (Fig. 4). In contrast, in Halothane-, Isoflurane-, and Pentobarbital-anesthetized mice, sometimes no increase at all could be observed but rather a continuous decrease of perfusion/mmHg from the very beginning until the end of the experiments. These findings indicate that Halothane, Isoflurane as well as Pentobarbital abrogate the vasoreactivity of brain vessels in spontaneously breathing mice and are therefore unsuited for CA measurements in this specie.

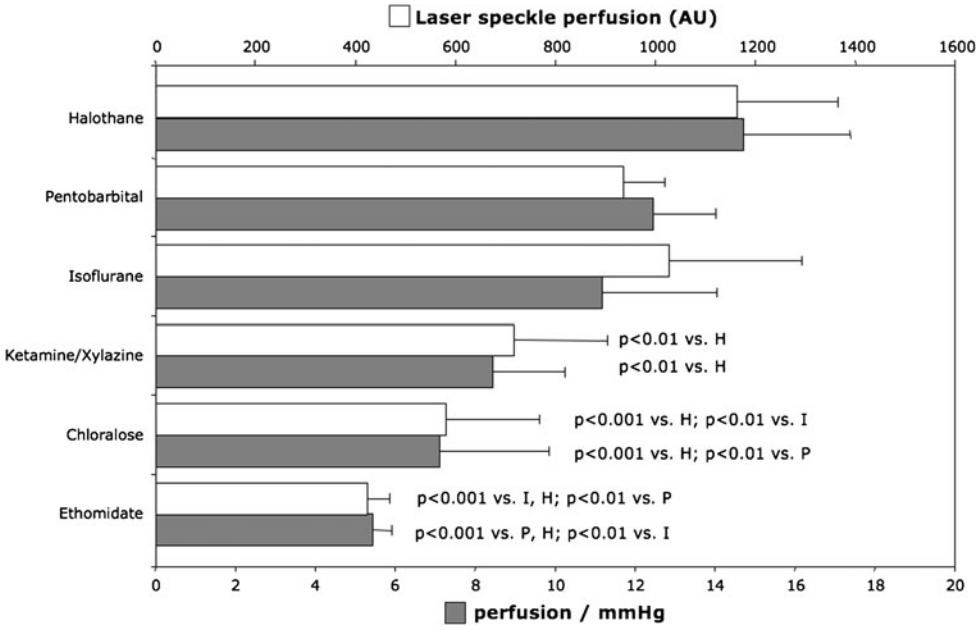

Fig. 3 Initial laser speckle perfusion (*white bars*) and initial perfusion normalized to the mean arterial blood pressure (*gray bars*). These data should be indicative for the vasodilatation state induced by the different anesthetics. Halothane, Isoflurane, and Pentobarbital that abrogated CA have the highest initial laser speckle perfusion as well as perfusion/mmHg values. *I* Isoflurane, *P* Pentobarbital, *H* Halothane

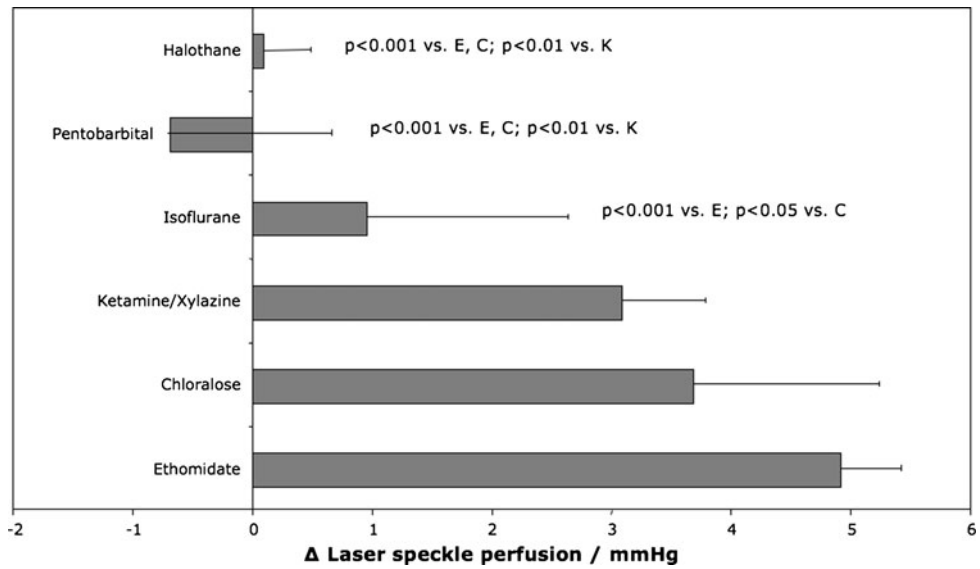

Fig. 4 Difference between the initial perfusion/mmHg and the maximal perfusion/mmHg. In contrast to Ethomidate, Ketamine/Xylazine and Chloralose anesthetized mice in Halothane, Pentobarbital and Isoflurane anesthetized mice more (Halothane) or less often (Isoflurane) no increase at all from the initial perfusion/mmHg value could be observed. In such a case, the perfusion/mmHg value at a blood pressure of 50 mmHg was taken. These data indicate that the highest vasodilatation reserve is present during Ethomidate anesthesia. *E* Ethomidate, *K* Ketamine/Xylazine, *C* Chloralose

Discussion

The present study clearly indicates that in spontaneously breathing mice, the anesthetic protocol for measurement of CA affects both the absolute values as well as the reproducibility of the measurements. In view of the high value of transgenic mice which are often also costly pre-treated before CA is finally measured, good reproducibility is an important point in choosing the anesthetic protocol to detect small but nevertheless biologic relevant differences between experimental groups. Here, we show that Ethomidate has an excellent reproducibility and retains reactivity of the cerebral vasculature best. Therefore, from all anesthetics tested in the present study, Ethomidate appears to be the anesthetic of choice for the measurement of CA in spontaneously breathing mice.

The best way to exclude any influence of anesthetics on mice appears to measure CA in the conscious state (Lacombe et al. 2005; Joutel et al. 2010). In these papers, the perfusion was quantified with Laser Doppler flowmetry via optical fibers glued to the scull of mice also equipped with catheters inside the femoral vessels. The animals had been placed in restrainers for 2 h before starting the final measurements. Laser speckle perfusion imaging that has been used in the present study, however, requires the scull to be fixed since it cannot distinguish tissue from red cell movements. In addition, restraining animals equipped with optical probes at the scull and catheters inside the femoral

vessels, even when treated with local anesthetics, for such a long period with final exsanguinations in the conscious state is difficult to get permitted in countries with strong animal protection laws such as Switzerland. Nevertheless CA measurements in conscious mice might allow assessing where the real limits are lying in this specie. In the aforementioned papers, the CA limits were determined in a different way when compared to the present study. These authors defined the lower limit as 90% and the upper limit as 110% of the initial blood flow. This way they obtained in wt mice a lower limit of about 60 mmHg, a value that is 15–20 mmHg above those reported by others for Ketamine/Xylazine and Chloralose (Niwa et al. 2002; Ayata et al. 2004; Tonnesen et al. 2005; Rosengarten et al. 2006) and that of the present study using the same anesthetics. However, when trying to apply the method for calculating the lower limit used in the present study (Pedersen et al. 2003) to the data of Lacombe et al. (2005) and Joutel et al. (2010) a lower limit of about 40–45 mmHg appears to be present also in their data. Thus, the lower CA limit in conscious mice is most close to our values and those of others obtained during Ketamine/Xylazine and Chloralose anesthesia. In Ethomidate-anesthetized mice, however, the lower limit is clearly lower, although there was only minor significance to the other anesthetics most likely due to much high data scatter in the Ketamine-/Xylazine- and Chloralose-anesthetized groups (cf. coefficients of variation, Fig. 2a). Concerning the upper limit, Lacombe et al. (2005) found during phenylepinephrine infusion and using their 110% method a value of 120 mmHg. Again when applying the method of the present study for determining the upper limit to their data the upper limit appears to be different, namely about 90 mmHg. Thus, the range of CA in mice appears to be about 40 mmHg. In naïve mice, a similar CA range was found only for Ethomidate-anesthetized mice, whereas in Ketamine-/Xylazine- and Chloralose-anesthetized mice, the range was 4- and 2-times smaller, respectively. In contrast, in phenylepinephrine treated mice, the CA range was not different between the different anesthetic protocols and with 35 mmHg also comparable to that found in naïve Ethomidate-anesthetized mice and that estimated from the data of conscious mice (Lacombe et al. 2005). Thus, Ethomidate appears to preserve the CA range best but to shift the autoregulation curve slightly leftward. In contrast, in naïve Ketamine-/Xylazine-and Chloralose-anesthetized mice, the CA range was clearly reduced suggesting an impaired vaso-reactivity that is also evident from the reduced maximal perfusion change per mmHg in response to the blood pressure drop (cf. Fig. 4). This is also in line with the lowest initial perfusion per mmHg in Ethomidate anesthesia (cf. Fig. 3), indicating a higher initial cerebrovascular constriction state and thus a higher vasodilatatory reserve.

Concerning Halothane only studies in rats could be found (Verhaegen et al. 1993; Pedersen et al. 2003), but Halothane slowly disappears from the market. In principle, the pharmacological action of Halothane is similar to that of Isoflurane but the wash-in and -out of Isoflurane is faster (Yasuda et al. 1989) enabling better control of the anesthesia. Thus, in recent years, Isoflurane became the standard volatile anesthetic also in animal research and was used in rats and mice for the measurement of cerebral blood flow under different conditions including functional stimulation (Ayata et al. 2004; Joutel et al. 2010) and cerebral autoregulation (Ayata et al. 2004; Tonnesen et al. 2005). One paper directly compared in mice Isoflurane with Choralose concerning CA as well as functional stimulation (Ayata et al. 2004) and found according to our results an abrogation of CA during Isoflurane anesthesia. In contrast, functional stimulation was not different between Isoflurane and Chloralose that is in line with the data of others also reporting in mice a preserved functional stimulation during Isoflurane anesthesia (Lacombe et al. 2005; Joutel et al. 2010) at an inspired concentration of 1.2–1.5%. We did not yet test Isoflurane and functional stimulation in mice, but previously in a study on rats, we used Ethomidate (Vogel and Kuschinsky 1996) because any functional stimulation-related CBF effect was abolished during Halothane anesthesia. This is in line with studies showing that the reactivity of the smooth muscle is impaired by volatile anesthetics (Bosnjak et al. 1992) as well with the data of the present study showing that Isoflurane like Halothane results in a complete loss of CA (cf. Fig. 1). However, a study dealing with the orthostatic regulation of mice claimed that CA was preserved during 1% Isoflurane anesthesia (Foley et al. 2005). Without measuring CA directly, this was concluded from the fact that phenylepinephrine increased mean arterial blood pressure but not CBF. However, phenylepinephrine results in a considerable tachycardia (in the present study by 61%) that in turn impairs cardiac output. Thus, an increased mean arterial blood pressure during phenylepinephrine treatment paralleled by a reduced cardiac output might leave CBF unchanged independent of an autoregulatory action of the cerebral vasculature.

The most significant problem when using Pentobarbital in spontaneously breathing animals is ventilatory depression that results in hypercapnia induced cerebral vasodilatation and subsequent loss of CA (cf. Table 2 and Fig. 1). To our knowledge, it has not yet been shown whether Barbiturates have also direct (pCO_2-independent) effects on CA, but dosages of pentobarbital previously published for paralyzed and artificially ventilated rats (Paterno et al. 2000) were not sufficient to keep our mice in the tolerance state. Consequently, we had to increase the dosage resulting in the highest arterial pCO_2 values of all groups. The present study was designed to test the effects of the different anesthetic

protocols in spontaneously breathing mice, because the great disadvantage of artificial ventilation is additional trauma and the necessity of several determinations of the arterial blood gases to adjust the respirator. Each blood gas determination requires at least about 100 µl of arterial blood. Thus, 2–3 determinations of blood gases can bring the animals close to a hemorrhagic shock. Although the lost blood can be replaced, e.g., by saline, this reduces the hematocrit and consequently the oxygen transport capacity of the blood that in turn increases CBF (Waschke et al. 1994). Of note, we supplemented the mice during the experiments with pure oxygen. In contrast to the vasodilating effect of hypoxia, CA is not different in man breathing either normal air or pure oxygen (Ogoh et al. 2010). In line with this study, we found no difference in the Laser speckle signal when switching from air to pure oxygen.

Using anesthetics for CA measurements has the principle and unavoidable disadvantage of influencing CBF and thus the CA measurement itself. In addition, anesthesia-induced hypercapnia might disturb CA. The same hold for intermittent sometimes un-recognized hypoxia that can occur during anesthesia especially when induced by injections. Since the brain is very sensitive to shortage of oxygen hyperoxia has been found to be very effective as emergency treatment in traumatic brain injury or stroke (Kumaria and Tolias 2009). Because it drastically reduces the risk of hypoxia during anesthesia but does not alter brain perfusion (cf. previous paragraph), we recommend supplying the mice with pure oxygen during surgery and subsequent CA measurements. Although hypercapnia and hypoxia can be avoided by artificial ventilation, this requires several determinations of the arterial blood gases, in mice easily resulting in a "diagnostic hemorrhage". In addition, one should be aware that transferability of data from animal experiments to the clinics could be hampered by the fact that different researchers perform CA measurements in a different way. One underestimated difference between protocols is anesthesia. Here, we aimed to find an anesthetic protocol that requires as little surgery as possible, to allow measurements in spontaneously breathing animals and has a high reproducibility. Such an anesthetic protocol reduces uncontrolled influences of side parameters on the data and thus increased the transferability of the data into clinics. Reproducibility is also a very important point in light of using valuable genetically modified mice that might be available only in limited numbers e.g. due to reduced breeding success. In this context also small systematic errors can be tolerated as long as the good reproducibility exceeds this disadvantage. Taken together, we feel that Ethomidate is—of the anesthetics tested here—the best due to its ability to keep the arterial pCO_2 in the normal range without the need of artificial ventilation, its excellent reproducibility, and a CA range closest to that of conscious animals (Lacombe et al. 2005; Joutel et al. 2010). The fact that Ethomidate shifts the CA curve slightly leftward could even be interpreted as an advantage, since this allows catching also the upper CA limit without phenylepinephrine treatment.

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Conflict of interest The authors declare no conflicts of interest.

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